27254-Ex3 Biofilm development and conjugative pili

Introduction

Many isolated plasmids carry the genetic information that promotes their self-transfer to other bacterial cells by means of bacterial conjugation. Conjugative plasmids belonging to the IncF incompatibility group are frequently isolated from *Enterobacteriaceae*, and in particular about 35 to 45 % of natural *E. coli* isolates carry such plasmids. Numerous virulence factors and antibiotic resistance genes are associated with this class of mobile genetic elements.

The conjugative IncF plasmids encode a specific type of transport machinery necessary for DNA transfer that includes a ~ 1-2 µm long, flexible proteinaceous pilus. This pilus is required for conjugation and is thought to mediate initial cell-cell contact between the plasmid carrying donor cell and the recipient cell. Expression of the conjugation machinery, including pilus production, is normally tightly regulated (presumably to reduce the cost for the host cell). Thus, under standard *in vitro* conditions in complex medium, only about 1 out of 1000 *E. coli* cells carrying a naturally repressed plasmid (e.g. R1) express a functional pilus and are therefore conjugation proficient. Several mutant plasmids which express pili constitutively (e.g. R1*drd*19) have been isolated *in vitro*. However, also normally repressed plasmids are thought to have the capability to derepress pili expression. This phenomenon of transient derepression of pili expression occurs immediately after one (infrequent) initial transfer. Possibly due to a slow establishment of a functional repression system in the plasmid-receiving cell (transconjugant), pilus expression is derepressed and the plasmid can thereby spread throughout a recipient population.

The need for cell-cell contact in the conjugation process implies that this type of gene transfer is most optimal in relatively dense populations, where the distances between the individual cells are small. It has consequently been of interest to investigate plasmid transfer in bacterial surface communities (biofilms), which are wide-spread in natural environments as the dominant mode of bacterial occurrence, and in which the bacteria are known to be positioned much closer to each other than in cell suspensions. In recent years the development of advanced microscopy and an increasing number of useful molecular tools for fluorescence tagging bacteria has opened a number of possibilities of tracking bacteria and specific DNA sequences such as plasmids in complex mixed cell populations. In this way it has been possible to map specific positions in a complex community at which plasmid transfer may be particularly intense.

Evidence has recently been presented that the presence of actively conjugating plasmids in *E. coli* cells induce biofilm development, and that this activity is coupled to the synthesis of conjugation pili attached to the bacterial cell surface. This could mean that plasmid transfer may be an auto-stimulatory process, which is initiated in cases where bacterial cells are close to each other (often the case on surfaces), and which subsequently induces biofilm development resulting in improved conditions for plasmid transfer.

The finding of a connection between bacterial conjugation and biofilm formation suggests that medically relevant plasmid-bearing strains are more likely to form a biofilm. This may influence both the chances of biofilm-related infection risks, and of conjugational spread of virulence factors and antibiotic resistance genes.

Aims

This exercise is designed to demonstrate that expression of conjugative pili promotes biofilm formation of *Escherichia coli* lab strains on plastic and glass surfaces. It will furthermore be tested whether additional factors that are thought to be important for *E. coli* K-12 biofilm formation, such as type I pili, flagella, or Ag43, are required for this plasmid mediated effect. Moreover, an experiment will be performed to investigate if, under conditions that support transient derepression, also repressed plasmids (such as R1) have the capability of promoting biofilm formation. Finally, an in situ investigation of plasmid transfer between bacteria on agar surfaces is performed.

Experimental 3 Procedures Overview

3A Construction of test strains by bacterial conjugation in suspension

Day 3
Preparation of cultures
4/1

Day 4
Conjugation and plating of transformants
5/1

Day 5
Count doner and transconjugants
6/1

3B Microtitre Tray Biofilm

Day 16
Preparation of cultures
17/1

Day 17
Inoculation of Microtitre plates
18/1

Day 18
Crystal violet staining (late in the day)
19/1

3C Flow chamber Biofilm

Day 11
Assembly of biofilm setup. Prepare the medium
12/1

Day 12
Assembly of biofilm setup. Prepare the medium (continued)
13/1

Day 15
Inoculation of biofilm setup with mutants and controls
16/1

Day 16+17+18
Inspect biofilm and record SCLM images
Analyze biofilm images using Comstat
17-19/1

3D Plasmid Transfer by conjugation on agar surfaces

Day 15
Preparation of cultures
16/1

Day 16
Conjugation and plating of transformants
17/1

Day 17
Inspection of plate conjugation using epifluorescence microscopy
18/1

3A: Construction of test strains by bacterial conjugation in suspension

Aim: Transfer of R1 plasmid to MG1655 wild type wt (sm^R); transfer of R1*drd*19 plasmid to MG1655 wt (sm^R), Δ*flhDC,* Δ*fim,* and Δ*flu* mutants (lacking flagella, type I fimbriae and Antigen 43 expression).

Day 3 (4/1) Preparation of cultures

You will receive plates with the strains written in the box blow. Pick a single colony from each plate and inoculate the recipient strains in 10 ml LB medium, and the donor strains in LB+ Km^{50}. Incubate the tubes with shaking overnight at 37ºC.

Donor strains (all *lac*-)	Recipient strains (all *lac*+)
D_1: SAR18 [R1] **D_2**: SAR18 [R1*drd*19]	**R_1**: MG1655 wildtype (Sm^R) **R_2**: MG1655 Δ*flhDC* **R_3**: MG1655 Δ*fim* **R_4**: MG1655 Δ*flu*

Day 4 (5/1) Conjugation and plating of transformants

1. Harvest 100 µL of donor cells by brief centrifugation at 10.000 rpm for 1 min. Resuspend in 1 mL fresh LB medium each (for removal of antibiotics).

2. Prepare 5 tubes with 900 µL LB medium and add 5 µL of resuspended D_1 to one tube and of resuspended D_2 to the remaining four tubes.

3. Incubate at 37°C without shaking for one hour (for expression of pili without shearing them off).

4. Add 100 µL of corresponding recipient cultures to the donor suspensions (R_1 to D_1, R_1-R_4 to D_2). Mix by inverting the tube several times (vigorous vortex could break pili).

5. Incubate for 40 minutes at 37°C without shaking. Vortex the mixtures for 30 sec and prepare proper dilutions in 0.9 % NaCl solutions (D_1R_1, D_2R_1 to D_2R_4 up to 10^{-5}).

Plating

1. Spread 100 µL of the 10^0, 10^{-1}, and 10^{-2} dilutions of D_1R_1 on LB /$Sm^{100}Km^{50}$ agar plates (only transconjugants can grow.) and 100 µL of the 10^{-3}, and 10^{-4}, and 10^{-5} dilutions of D_1R_1 on LB /Km^{50} agar plates (donors and transconjugants can grow).

2. Spread 100 µL of the 10^{-3}, 10^{-4}, and 10^{-5} dilutions of D_2R_1 to D_2R_4 on MacConkey /Km^{50} plates (donors and transconjugants can grow but can be differentiated by their *lac* phenotype). Incubate at 37°C over night.

Day 5 (6/1)

Count donors and transconjugants on plates incubated on day 4 separately to calculate the transfer frequency (transconjugants/donor ratio). Store the plates at 4°C fridge. Transconjugants (red colonies) will be used in part B.

3B: Biofilm formation by *E. coli* K-12 strains in microtiter plates

Aim:

- Investigations of roles of plasmid R1 and R1*drd*19 in biofilm formation by MG1655 wt and SAR18.
- Investigations of roles of Δ*flhDC,* Δ*fim,* and Δ*flu* mutations on biofilm induction by R1*drd*19 in MG1655.
- Testing if mixing of SAR18 carrying R1 with the plasmid-free strain induces biofilm formation (potentially by means of transient derepression).

Day 16 (17/1) Inoculation of cultures

Prepare overnight cultures of the following strains for Part B experiments in LB (LB+ Km^{50} for plasmid carrying strains).

T_1: MG1655 wildtype (Sm^R) **T_2**: MG1655 wildtype (Sm^R) [R1] **T_3**: MG1655 wildtype (Sm^R) [R1 *drd*19] **T_4**: MG1655 Δ*flhDC* [R1*drd*19] **T_5**: MG1655 Δ*fim* [R1*drd*19] **T_6**: MG1655 Δ*flu* [R1*drd*19]	**T_7**: SAR18 **T_8**: SAR18 [R1] **T_9**: SAR18 [R1*drd*19]

Day 17 (18/1) Microtitre Plate Biofilm Inoculation

1. Harvest 100 µL of all test strains by brief centrifugation at 10.000 rpm for 1 min.
2. Resuspend in 1 mL fresh LB medium each (for removal of antibiotics).
3. Dilute resuspended cells of T_1 – T_9 into 1ml fresh LB to an OD_{600} of 0.02 (no antibiotics need to be added for plasmid propagation).
4. Leave all the border wells for sterile medium as control (this strategy also help to avoid evaporation).
5. Fill 100 µL of the dilutions in wells of a Falcon PVC 96-well plate (at least 3 wells per test strain).
6. In addition, mix equal volumes of the dilutions of T_7 with T_8 and T_7 with T_9. Proceed with the mixtures as with the previous dilutions.

7. Incubate the 96- well plate in a sealed plastic bag (in order to avoid evaporation) at 37°C for 24 hours.

Day 18 (19/1) Microtitre Plate Biofilm Analysis

1. Check growth in the inoculated wells.

2. Remove the suspended cells (put the microtitre plate upside down quickly for 2-3 times on the top of a yellow bag). Wash the biofilms with 2 x 130 µL 0.9 % NaCl solution.

3. Add 130 µL of 0.1 % Crystal Violet solution. Incubate for 15 min at room temperature.

4. Wash the stained biofilm as before (2 x 140 µL 0.9 % NaCl solution). After the last wash, put the microtitre plate upside down on a towel paper for a couple of times, in order to get rid of the background.

5. Finally, add 150 µL of ethanol and read A_{590} after 30 minutes. Dilute the solubilized Crystal Violet solutions if initial A_{590} reading is above 2.5 and measure absorption again. Subtract the average reading of the control wells from the average reading of the inoculated wells of the relevant test strain.

Note:

Wear gloves!

Pour crystal violet in a yellow bag. Not in the sink! Beware of splashes – it can not come of your clothes!

3C: Biofilm formation by *E. coli* K-12 strains in flow chambers

Aim: In this part of the exercise we will compare structure development of four biofilms: *E. coli* SAR18 (wt), *E. coli* SAR18 [R1*drd*19], a 1:1 mixture of *E. coli* SAR18 and *E. coli* SAR18 [R1*drd*19], and a 1:1 mixture of *E. coli* SAR18 and *E. coli* SAR18 [R1]. All the employed strains have been *gfp* tagged for visualisation of the biofilm structure by confocal microscopy.

The investigation of the mixed biofilms will be divided among the teams, so each team only need to make one of them and then exchange the results with another group. The teams with an even number will investigate the 1:1 mixture of *E. coli* SAR18 and *E. coli* SAR18 [R1] and the odd numbered teams will investigate the 1:1 mixture of *E. coli* SAR18 and *E. coli* SAR18 [R1*drd*19].

Day 11 (12/1) Assembly of biofilm system and preparation of medium

1. Build up the biofilm system, by following the biofilm protocol in appendix A. Glue flowcells and assemble bubble traps; connect tubings with bubble traps, flowcells and the pump. Let stand overnight for flowcells to dry.
2. Prepare 9L FB medium as described in appendix A. Autoclave the medium.

Day 12 (13/1) Assembly of biofilm system and prepare the medium (continued)

1. Finish the assembly of the biofilm system.
2. Sterilize the biofilm system for 3-4 hours using a 0.5% hypochlorite solution (***use gloves and glasses***). Wash the biofilm-system with sterilised water for 1-2 hours.
3. Add 1L A10, 9ml 20%glucose, 10ml 1µg/ml thiamine and 10ml 10µg/ml proline into 9L autoclaved FB medium. Handle sterilely!
4. Run media through the biofilm system at 0.5 rpm at 30ºC (over the weekend).

Day 10 (15/1) *(the teacher will do this)*

Inoculate broths with SAR18, SAR18 [R1], SAR18 [R1*drd*19] in 10ml LB Km ^{50} and incubate overnight at 30ºC.

Day 11 (16/1) Inoculation of the biofilm system

1. Dilute the overnight culture to OD_{600}=0,01 in 0.9%NaCl. Inoculate the flow channels with the *E. coli* strains SAR18 (wt), SAR18 [R1*drd*19], and a 1:1 mixture of *E. coli* SAR18 and *E. coli* SAR18 [R1*drd*19] or a 1:1 mixture of *E. coli* SAR18 and *E. coli* SAR18 [R1] as described in appendix A (will also be demonstrated).
2. The biofilm system should hereafter be kept at 30ºC until visualization using the confocal microscope.

Day 16-18 (17-19/1) Inspection of biofilms and recording CLSM images. Analysis of biofilm images by the use of Comstat

Using the confocal microscope, inspect the biofilms; look for differences and/or similarities and record pictures for later image processing and quantification by COMSTAT. In order to get representative data, take at least four image stacks of each biofilm randomly down the channel (normally if time permit, it requires at least six image stacks for COMSTAT analysis), using the 40x objective.

In the end of the day transfer all the images that were acquired during the last couple of days to the scope2/student_kusus27254 folder. Process images using Imaris software and quantify the biofilms using the COMSTAT software (see appendix about COMSTAT). The analysis of the biofilm images results in a range of different variables such as *average thickness*, *roughness*, *surface to volume ratio*, *substratum coverage* etc. Collect and evaluate all the results, also from the other teams, and try to visualize differences or similarities between the different biofilms using e.g. MS Excel.

3D: Plasmid transfer by conjugation on agar surfaces

Day 15 (16/1) Inoculation of cultures

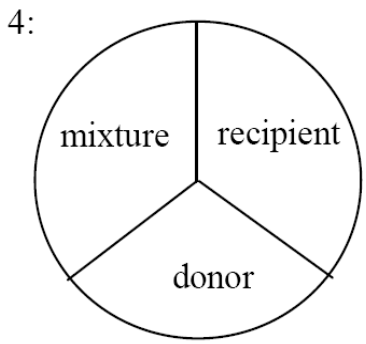
In this part of the exercise you are going to perform an *in situ* investigation of plasmid transfer on agar surfaces. *Pseudomonas putida* R1 is used as the recipient strain and *P. putida* KT2442, harboring the TOL plasmid, is used as donor.

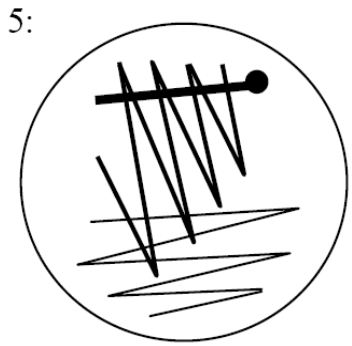
You are going to perform the experiments as indicated in the scheme below. Prepare overnight cultures in LB of the following strains:

Donor	Recipient
P. putida KT2442 *lacI*^{q}/TOL::$P_{A1\text{-}04/03}$-Gfp (pSM1806)	*P. putida* R1 *rrnB*P1-*rfp* (SM1877)

Day 16 (17/1) Preparation of plate conjugation

1. In order to follow the conjugation and mobilization on agar surfaces, dry 4 LB plates, two for each of the above transfer experiments.
2. Wash 1ml donor and recipient twice in 0.9% NaCl and resuspend in 1 ml 0.9% NaCl.
3. Now mix 25 µl of donor and recipient in a new eppendorf tube (doner 1 + recipient1, doner2 + recipient 2).
4. For each of the transfer mixtures add 1 droplet of 2 µl on a LB plate; add also 2 µl washed donor and recipient cells (washed separately) on the same plate (divide the plate into three). Use the P20 Gilson pipette. Let dry.
5. Place a 5 µl droplet from each of the transfer mixtures near the edge on the other LB plate. Streak in the same way you streak for single colonies.
6. Incubate all 4 plates at 30ºC overnight.





Day 17 (18/1)

Look at the spots on the LB plates in the fluorescent microscope. Leave the plates at the table for further incubation.

Take pictures today or tomorrow dependent on the result you observe on the plates (maybe the plates have to be incubated longer before the results are clear). Use the CCD camera on the epifluorescent microscope. Look at the spots on the LB plates in the microscope again next day.

References

1. Reisner A, Haagensen JA, Schembri MA, Zechner EL, Molin S. 2003. Development and maturation of Escherichia coli K-12 biofilms. *Mol Microbiol* 48:933-46

2. Haagensen JA, Hansen SK, Johansen T, Molin S. 2002. In situ detection of horizontal transfer of mobile genetic elements. *FEMS Microbiology Ecology* 42:261-8